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Flow cytometry techniques in radiation biology

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SUMMARY

Hematopoietic stem cells (HSC) are present in the marrow at a concentration of approximately 2–3 HSC per 1000 nucleated marrow cells. In the past, only clonogenic assays requiring 8–13 days and ten irradiated recipient rodents were available for assaying HSC. Because of the importance of HSC in the postirradiation syndrome, we have developed a new rapid method based on flow cytometry not only to assay but also to purify and characterize HSC. This new method makes extensive use of monoclonal antibodies conjugated to fluorescent phycobiliproteins through the sulfhydryls of the hinge region of the IgG molecule. An optical bench arrangement with a dye laser and an argon laser was used for dual excitation of the phycobiliprotein-monoclonal antibody conjugates and various cellular and DNA probes. Using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) exclusion to identify viable cells, it was possible to follow regeneration of postirradiated rat marrow HSC.

INTRODUCTION

Considerable evidence exists that all blood cells are derived from HSC. These cells are of interest to radiobiologists because they are highly sensitive to low doses of ionizing radiation [1]. In the rodent HSC, as measured by the CFU-S assay, are characterized by a D_0 of 0.8–1.0 Gy. As such, doses greater than 9 Gy effectively

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Abbreviations: HSC, hematopoietic stem cells; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FITC, fluorescein isothiocyanate; PhyB, phycoerythrin B; PhyR, phycoerythrin R; APC, allophycocyanin; MMB, maleimidobutyryl biocytin; TBR, total body irradiation; FDA, fluorescein diacetate; PI, propidium iodide; CFU, colony-forming unit; FACS, fluorescence-activated cell sorting; UV, ultraviolet; NBR, Norway Black Rat; WGA, wheat germ lectin; PMT, photomultiplier tube.

sterilize the HSC compartment. If not rescued by bone marrow grafting, the lethally irradiated individual will eventually die, primarily from bleeding and sepsis, due to the inability to replenish blood cells from HSC. Therefore, it is of importance to understand how radiation damages these HSC in order to prevent this damage or to devise methods by which the damaged HSC may be replaced in the irradiated individual.

The major difficulty in studying directly the interaction of ionizing radiation with the HSC is the scarcity of these cells in the marrow and the lack of a distinguishing feature necessary for their accurate and rapid identification. In the rat, HSC comprise less than 0.3% of the total marrow hematopoietic cells [2,3]. In the past, efforts to isolate the HSC used physical techniques such as density gradient centrifugation in isotonic media, sedimentation velocity, elutriation, electrophoresis, nylon wood filtration, and plastic adherence. These techniques usually result in purification of less than 10-fold, which is well short of the 300-fold-plus

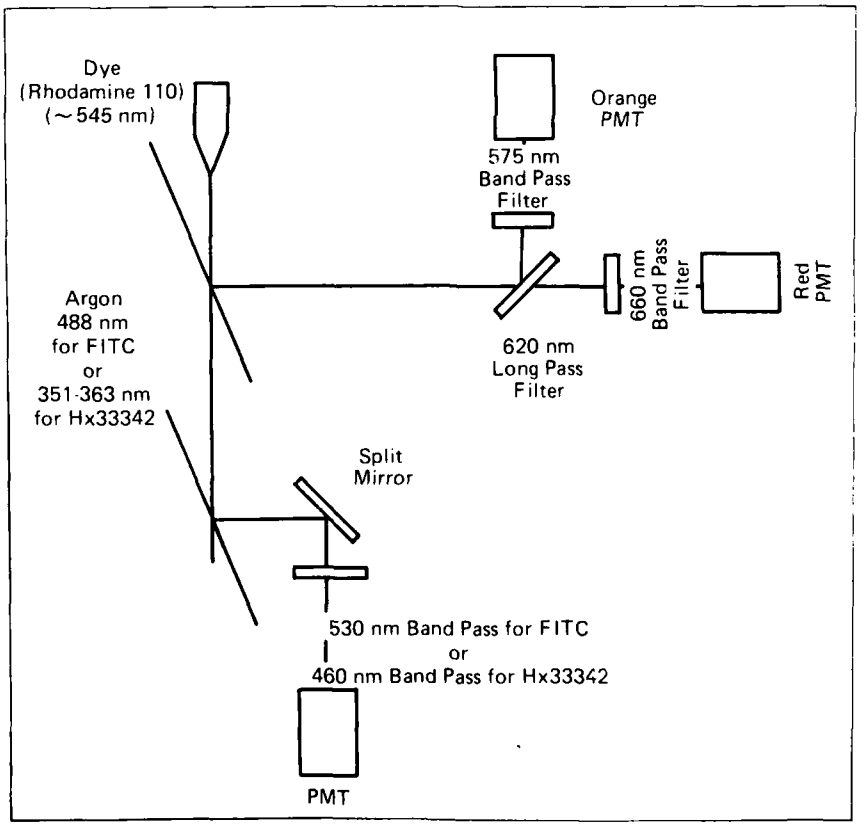

Fig. 1. Optical bench arrangement of 4-parameter light-activated cell sorter.

purification necessary to generate a pure HSC population. For this reason this laboratory and others [4] have made extensive use of the techniques of flow cytometry and/or fluorescence-activated cell sorting (FACS) [5] for the isolation and identification of HSC.

MATERIALS AND METHODS

Flow cytometry and sorting

Flow cytometry measurements and cell sorts were performed on a dual laser Becton Dickinson FACS II cell sorter interfaced to a Consort 40 computer system. The optical bench arrangement of the dual lasers is shown in Fig. 1.

Phycobiliproteins

Studies on the isolation and characterization of HSC have made extensive use of a new class of fluorescent proteins referred to as phycobiliproteins [6]. These fluorescent proteins are isolated from cyanobacteria (blue-green algae) and red algae. They show maximum absorbance in the 470–650 nm region, and fluorescence emission bands begin in the orange (550 nm) and extend into the far red. The molar fluorescence intensity of phycoerythrins is up to 20 times that of fluorescein isothiocyanate (FITC). Several different proteins are included in the phycobiliprotein family. We have used primarily phycoerythrin B (PhyB) and allophycocyanin (APC). The excitation and emission spectra of these two phycobiliproteins are shown in Fig. 2.

Lasers and optical bench arrangement

Because both phycoerythrin R (PhyR) and FITC have an absorbance maximum between 490 and 496 nm but emit at different wavelengths (in green and orange, respectively), most dual parameter immunofluorescence work is performed using the strong 488.0 nm spectral line of the argon laser to excite both PhyR- and FITC-conjugated monoclonal antibodies bound to cell surface determinants. However, we have chosen to label two different monoclonals with two different phycobiliproteins rather than one phycobiliprotein and FITC [3]. We have found that excitation for both APC and PhyB can be performed effectively with the krypton laser tuned to the 530.9 nm spectral line, by an argon laser tuned to the 528.7 nm spectral line, or by a dye laser (rhodamine 110) tuned to approximately 545 nm. Further, we have shown that APC can also be excited with the 488.0 argon laser spectral line, allowing 3-parameter immunofluorescence studies (APC, PhyB, and FITC) to be performed with a single laser. However, it was determined that the best results are obtained when the dye laser is used as the primary laser and the argon laser (tuned to the 488

nm or UV spectral lines) is used as the delayed or secondary laser. This optical bench arrangement allowed us to not only identify the HSC with the dye laser but also to analyze and characterize the HSC with the argon laser.

Conjugation of monoclonal antibodies to phycobiliproteins

It was found previously [2] that incubation of marrow cells with the monoclonal antibody Ox7 restricted the generation of splenic hematopoietic colonies (see section HSC assays), presumably due to initiation of in vivo cellular cytotoxic events by the Fc region of the bound IgG molecule. This problem can be avoided by digesting the

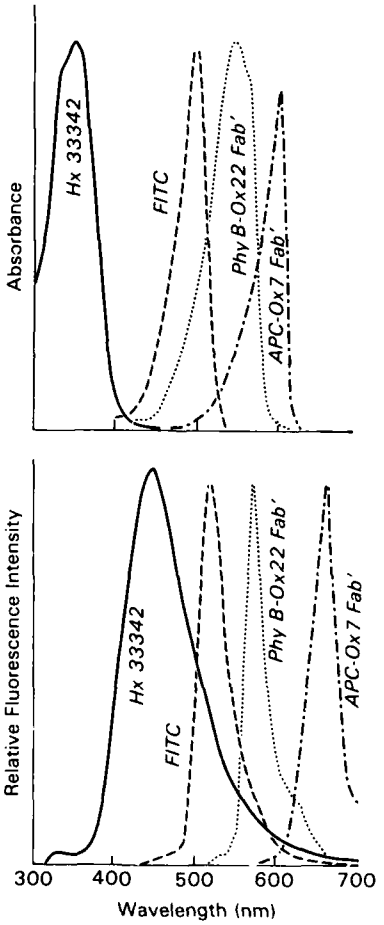

Fig. 2. Excitation and emission spectra of several compounds. Hx33342 is a DNA-specific dye used to generate cell cycle DNA histograms. Absorption and emission fluorescence spectra were determined on a model SLM-8000 fluorescence spectrophotometer. Spectra are normalized at peak amplitude and are uncorrected.

monoclonal antibody with pepsin, which produces the $F(ab')_2$ fragment. The $F(ab')_2$ fragment is then reduced with Cleland's reagent, and the Fab' with its free sulfhydryl is linked through a thioether bond to the maleimide-phycobiliprotein conjugate. Although time consuming, this technique has proven to be a reliable method of conjugating monoclonal IgG to phycobiliproteins. The following monoclonal antibodies have all been conjugated to either PhyR, PhyB, or APC using this technique: W3/13, Ox22, Ox7, W3/25, Ox19, Ox8, and My10 [2,3]. These conjugates generate a more intense signal than can be achieved using the sandwich technique, which uses FITC-antimouse IgG antibody as the second antibody. There are other benefits of this technique. The Fab'-phycobiliprotein conjugates have a long shelf life (greater than a year) when stored at 4°C in 0.01% sodium azide. There is an absence of binding to Fc receptors, they are stable when shipped through the mail, and it is easy to perform 3-parameter immunofluorescence studies by using FITC-antimouse IgG Fc-specific antibody as the second reagent in the sandwich technique [7].

A variation of the conjugation technique described above is as follows. Maleimidobutyryl biocytin (MMB) is substituted for the maleimidephycobiliprotein, the result being biotinylation of the free Fab' sulfhydryl group. Following incubation of cells with the biotinylated Fab' antibody, avidin conjugated to either FITC or phycobiliprotein is added [3]. Unfortunately, this technique does not work with all monoclonal antibodies. For example, it works well with Ox8 but not with W3/13 or Ox22.

Animals

Lewis rats (male, 6–8 weeks old) were obtained from Charles River Labs (Kingston, NY) and were quarantined on arrival and screened for evidence of disease before being released from quarantine. They were maintained in an AAALAC-accredited facility in plastic Micro-isolator cages on hardwood chip contact bedding and were provided with commercial rodent chow and acidified tap water ad libitum. The rats were on a 12 h light/dark full spectrum with no twilight. Total body irradiation (TBR) was delivered with an opposing ^{60}Co radiation source as described previously [3]. Rats were killed by exsanguination while under methoxyfurane inhalation anesthesia. Irradiated rats were given 1 mg/ml tetracycline in their drinking water. Rats were cared for according to principles enunciated in the Guide for the Care and Use of Laboratory Animals Resources, National Research Council.

HSC assays

Three different HSC assays were used to measure the degree of HSC purification. They were the CFU-S assay [2], the 30-day survival of lethally irradiated recipient

rats [3], and the intrathymic injection of histocompatible, T-cell allotype disparate donor stem cells [7]. The CFU-S assay was performed as follows: irradiated rats (9 Gy ^{60}Co TBR, 0.4 Gy/min) were grafted by intravenous injection of bone marrow cellular suspensions or purified HSC. In the rat, approximately 3–10% of the stem cells seed the spleen [2], proliferate, and form discrete colonies of hematopoietic cells. The number of colonies formed is proportional to the number of HSC injected. This clonogenic assay is cumbersome: 11–13 days, and a large number of irradiated recipient animals, are required to perform this assay. The second assay involves grafting of HSC in order to protect lethally irradiated rats from the lethal effects of irradiation. Rats receive a dose of 9.5 Gy ^{60}Co TBR, 0.4 Gy/min. The rats are then grafted with either normal marrow or purified HSC. The ratio number of normal marrow cells or purified HSC required to rescue the lethally irradiated rat is used to calculate the degree of purification of HSC. This assay is generally considered to be a more accurate assay for HSC than the CFU-S assay, which appears to measure committed as well as multipotent HSC [8]. The third assay used was the direct injection of either normal or purified Lewis rat HSC into the thymus of sublethally irradiated (8 Gy ^{60}Co TBR, 0.4 Gy/min) Norway Black Rat (NBR)

TABLE I

MONOCLONAL ANTIBODIES RECOGNIZING DETERMINANTS ON RAT LYMPHOCYTES

Monoclonal antibody[a]	Antigenic determinant recognized	Cell population recognized
Ox2	Glycoprotein on thymocyte membrane	B-cells, neuronal endothelial cells, thymocytes, dendritic cells
Ox3	Polymorphic determinant on rat Ia	B-cells, some epithelial cells, dendritic cells
Ox4	Polymorphic determinant on rat Ia	B-cells, 20% of thymocytes
Ox6	Monomorphic determinant on rat Ia	B-cells, 20% of thymocytes
Ox7	Rat Thy-1.1 antigen	Thymocytes, stem cells, neuronal cells, immature B-cells
Ox8	Glycoprotein determinant on thymocyte membranes	Cytotoxic, suppressor T-cells, NK, thymocytes
Ox12	Rat kappa chains	B-cells
Ox17	α-Chain rat Ia-antigen	B-cells
Ox18	Monomorphic determinant rat class I MHC	Class I MHC (RT-1A)
Ox19	Glycoprotein on thymocytes	Thymocytes, peripheral T-cells
Ox22	High molecular weight form of leukocyte common antigen	B-cell, some T-cells
W3/13	Sialoglycoprotein of rat thymocytes	Thymocytes, peripheral T-cells, granulocytes
W3/25	Glycoprotein on helper T-cells	Helper T-cells, thymocytes

[a]Mason et al. [17].

recipient rats [9]. After an extended incubation period, the HSC proliferate and differentiate into thymocytes. The number of newly generated thymocytes is measured by the use of the allogeneic system designated as RT 7. Lewis rats express the RT 7.1 antigen on thymocytes while the histocompatible NBR rats express the RT 7.2 antigen. Using the monoclonal antibodies BC-84 and 8G6.1, which recognize RT 7.1 and RT 7.2, respectively, the number of donor thymocytes (from Lewis marrow cells) that appear 18 days postinjection is a linear function of the number of cells injected. This assay establishes the origin of the newly formed thymocytes. Further, the thymus is an avascular organ and this minimizes the escape of the injected HSC into the circulation. The main advantage of this assay is its sensitivity, as it is 50 times more sensitive than the CFU-S assay [10].

Cell preparation and staining

Rat marrow cells were prepared as described previously [3]. Staining with fluorescein diacetate (FDA) was performed according to the Hale and McCarthy method [11]. Cells were tagged with FITC-wheat germ lectin (FITC-WGA) according to the method of Visser et al. [12]. DAPI (1 mg/ml in distilled water) and propidium iodide (PI) (1 mg/ml in ethanol) were added to a cell suspension at a final concentration of 10 μg/ml and 20 μg/ml, respectively.

RESULTS

HSC characterization and isolation

The ideal solution to measuring the concentration of marrow HSC is to develop a monoclonal antibody to a membrane antigenic determinant expressed only on the HSC and no other marrow cell. Conjugation of this antibody with the appropriate fluorochrome would then allow fluorochrome-tagged HSC to be identified and separated from the remainder of the marrow by cell sorting. Unfortunately, no such unique HSC antigen has been identified to date. In order to isolate the HSC, it was necessary to label marrow hematopoietic cells with a 'cocktail' of fluorochrome-conjugated monoclonal antibodies and/or lectins and to identify the HSC by pattern recognition. A partial list of monoclonals used and their specificities is presented in Table I.

The combination of conjugated antibodies that proved to be optimum for HSC identification was APC-Ox7 Fab', PhyB-Ox22 Fab', and FITC-W3/13 IgG. A correlated dual parameter plot of marrow cells tagged with the above-mentioned antibodies and excited with a dye laser (rhodamine 110) tuned to 545 nm is shown in Fig. 3. The HSC was found exclusively in the Ox7 upper 20% positive, Ox22 negative window [3,7]. The purification of HSC as measured by the CFU-S assay, survival following lethal irradiation, and intrathymic injection are presented in

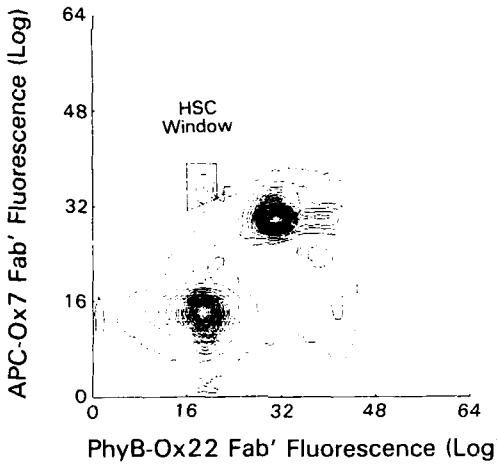

Fig. 3. Contour plot of rat marrow cells labeled with PhyB-Ox22 Fab' and APC-Ox7 Fab'. HSC were found only within the Ox7 upper 20% positive, Ox22 negative window and cells within this window accounted for 0.265% of the total marrow cell population.

Table II. There is a discrepancy between the CFU-S assay and the other two assays for measuring HSC purification. We believe this is due in part to an obligatory step of HSC lodgement in bone marrow before it can express itself in the spleen as a CFU-S assay [13]. Further, a more complex, dual laser, 3-parameter immunofluorescence study using a second argon laser tuned to 488 nm and FITC-W3/13 as the third antibody showed that the Ox7 upper 20% positive, Ox22 negative cells to be primarily W3/13 dim (80–90%) and the remainder W3/13 positive (data not shown).

Using the sandwich technique [7], it was possible to further characterize the HSC as being Ox8, W3/25, Ox6 and Ox17 negative; possibly Ox19 dim; and Ox18 very bright. The specificities of these monoclonal antibodies are given in Table I. The optical bench arrangement shown in Fig. 1 can also be used to characterize HSC with respect to lectin receptor sites and various intracellular enzyme systems. For example, the rat HSC binds FITC-WGA and is apparently characterized by a high cytoplasmic concentration of nonspecific esterases as measured by FDA uptake as shown in Figs. 4 and 5.

TABLE II

ASSAYS FOR ANALYZING PURIFICATION OF STEM CELL POPULATIONS

Route of injection	Assay system	Stem cell purification
i.v.	CFU-S	100
i.v.	Irradiated recipient survival	350
i.t.	Intrathymic adoptive transfer	282

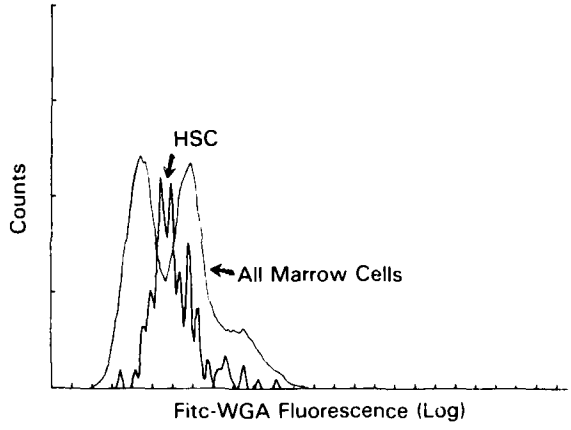

Fig. 4. Relative fluorescence distribution of marrow cells and HSC incubated with FITC-wheat germ agglutinin.

Comparison of dose survival curves generated by the CFU-S and FACS assays

HSC were assayed in irradiated rats by both the CFU-S and FACS procedures. These two assay systems generated different results as shown in Fig. 6 and reported previously [14]. One of several possible explanations for this discrepancy might be nonspecific tagging of dead and dying cells with the phycobiliprotein-monoclonal antibody conjugates so that these dead cells are scored as HSC by the FACS procedure, leading to overestimation of the concentration of viable HSC in the marrow

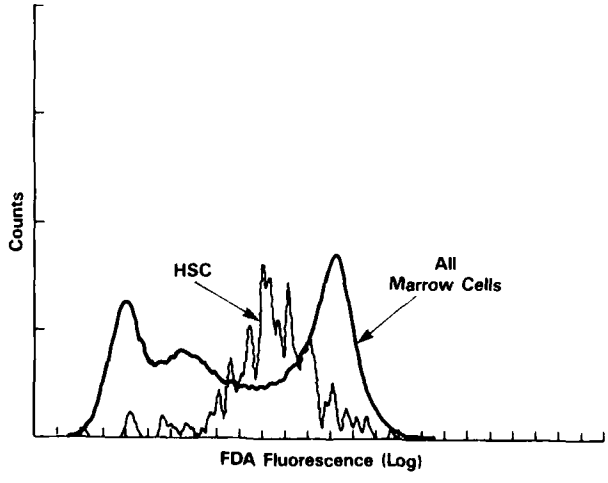

Fig. 5. Relative fluorescence distribution of marrow cells and HSC incubated with FDA.

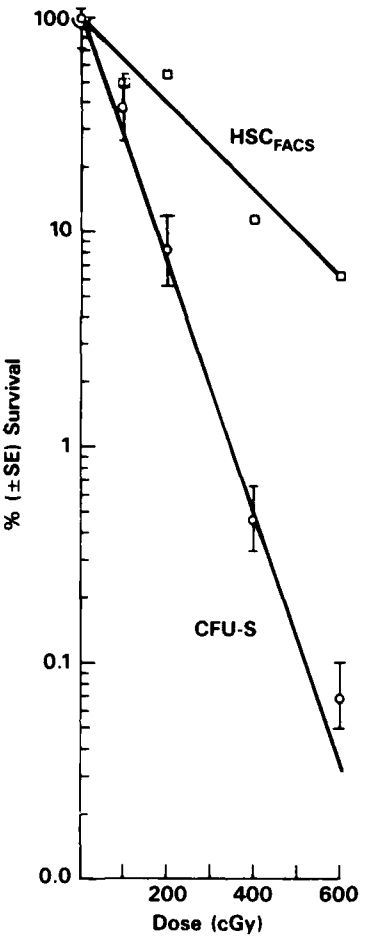

Fig. 6. Radiation dose-response curves for HSC as measured by FACS and CFU-S assays.

of postirradiated rats. Therefore, a new technique was devised for determining and enumerating viable marrow cells, including HSC, in the immediate postirradiated marrow.

DAPI staining of dead cells

The most commonly used fluorescent probe in flow cytometry for staining and gating out dead cells from immunofluorescence histograms is PI. It is an intercalating DNA dye with an absorbance maximum in the yellow and an emission maximum in the orange-red (for a more comprehensive discussion of DNA stains, see [15]). Dead cells are labeled with PI by including it in the last cell wash at a concentration of 20 μg/ml. Live cells exclude the PI while dead cells take up the stain. The

result is an intensive orange-red nuclear fluorescence emanating from the dead cells. Although the fluorescence of PI overlaps that of PhyB and PhyR, PI can be used to gate out dead cells in 2- and 3-parameter immunofluorescence studies. The orange-red fluorescence of the PI-stained dead cells is an order of magnitude brighter than the immunofluorescence from the PhyB- or PhyR-antibody-tagged viable cells and does not interfere with or alter the immunofluorescence histograms of viable cells. However, the immunofluorescence histograms of dead and dying cells cannot be determined in the presence of PI, for the PI fluorescence cannot be separated from the PhyB or the PhyR fluorescence. In order to determine the phenotype of dead cells it was necessary to use a fluorescent probe whose emission spectrum did not overlap that of FITC, Phy, or APC. Such a fluorescent probe is DAPI.

DAPI is a nonintercalating DNA-specific stain with an absorption maximum in the UV and an emission maximum in the blue [15]. Like Hx33342, DAPI can be excited with the argon laser tuned to the UV and the resulting fluorescence can be detected using the 460 nm PMT (Fig. 1). In order to demonstrate that DAPI stains only dead cells, 20 μg PI and 10 μg DAPI were added to a 1 ml solution of 5×10^6 rat marrow cells. The cells were allowed to incubate for 30 min at 4°C and then analyzed on the FACS. The PI-stained cells were excited with the dye laser and their fluorescence measured with the orange PMT, while DAPI-stained cells were excited with the UV laser and their fluorescence recorded on the violet-blue PMT. The correlated PI and DAPI fluorescences are plotted in Fig. 7. Clearly, marrow cells that stain with PI (i.e., dead cells) also stain with DAPI. Viable cells that do not stain with PI also exclude DAPI. Therefore, substituting DAPI for PI as a cell viability stain allows not only dead cells to be gated 'out' of 2-parameter im-

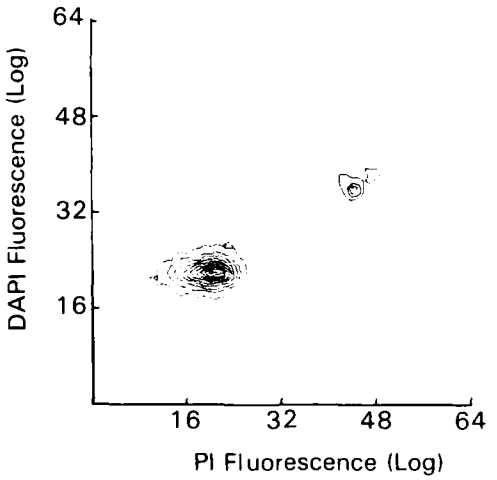

Fig. 7. Contour plot of rat marrow cells labeled with PI and DAPI.

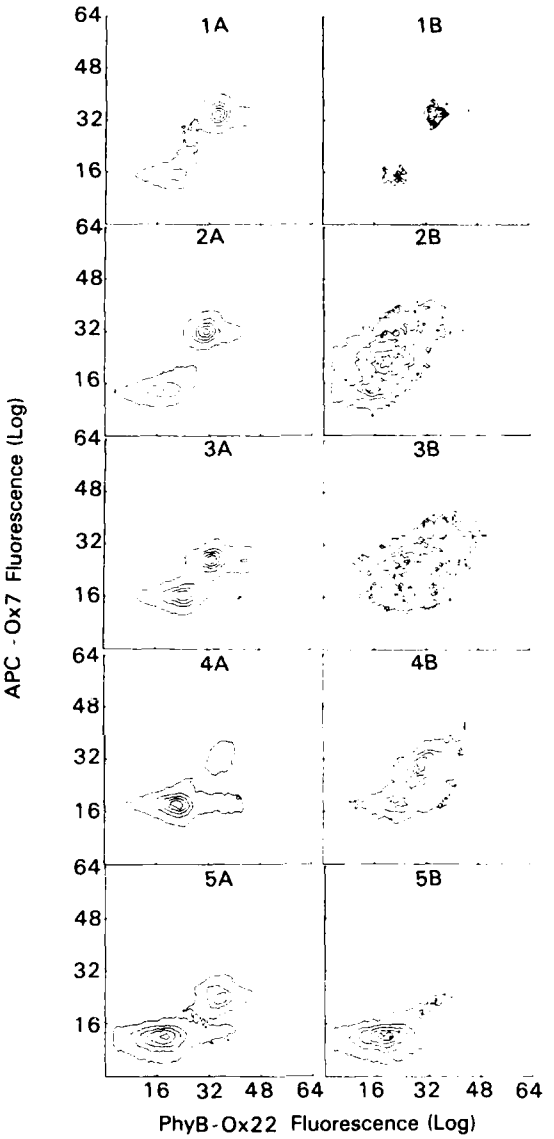

Fig. 8. Contour plots of viable (*A*) and dead (*B*) cells labeled with APC-Ox7 Fab', PhyB-Ox22 Fab', and DAPI. DAPI fluorescence was determined with the delayed or second laser and is not shown. Viable cells are DAPI negative while dead cells are DAPI positive. Time sequence from top to bottom is as follows: (1) normal marrow, (2) 3 h postirradiation, (3) 1 day postirradiation, (4) 3 days postirradiation, and (5) 5 days postirradiation. Contour maps were drawn as a percent of peak amplitude at increments of 5%, 25%, 45%, 65%, and 85%. Radiation was 2 Gy TBR ^{60}Co at a dose rate of 0.04 Gy/min.

munofluorescence work but also makes it possible to gate 'on' dead cells for the analysis of both the rate and phenotype of dying cells in the postirradiated rat marrow. Such a study is shown in Fig. 8, where the phenotypes of viable and dead cells are presented as a function of time postirradiation. The first cells to die immediately postirradiation appear to be blast cells and erythrocytic precursors, followed by lymphocytes at 1–3 days postirradiation and granulocytes at 4–5 days postirradiation. Further information on the dead cell fraction in the total marrow population and the dead cell fraction within the Ox7 upper 20% positive, Ox22 negative window or HSC window is given in Table III.

DISCUSSION

New techniques were developed for the identification of rat HSC in normal marrow by flow cytometry using a modified FACS-II instrument. When these techniques were used to monitor the concentration and regeneration of HSC in the postirradiated rat, discrepancies between the CFU-S assay and the FACS assay for determining the relative size of the postirradation HSC compartment became obvious. The results presented in the present study indicate that part of the difference

TABLE III

PERCENT DEAD CELLS INCLUDING HSC IN POSTIRRADIATED RAT MARROW

Experiment		Total cells/ femur × 10^{-7}	% Total cells DAPI$^+$	HSC/10^5 cells	% HSC DAPI$^+$	Corrected HSC/10^5 cells	HSC/femur
Control[a]							
	1	3.20	0.8	307	2.0	301	96 320
	2	4.80	0.5	408	0.2	407	195 360
Irradiated[b]							
3 h	1	2.30	23.3	1 071	63.4	392	90 409
	2	1.75	24.6	1 104	67.8	355	62 210
6 h	2	1.30	26.9	347	24.2	263	34 193
	2	1.10	23.9	416	23.8	317	34 869
24 h	1	0.80	4.7	463	14.7	395	31 595
	2	0.55	11.7	277	22.0	216	11 883
30 h	1	0.55	12.8	252	7.9	232	12 765
	2	0.32	17.6	250	16.0	210	6 720
3 days	1	2.70	19.3	78	2.6	75	20 512
	2	2.47	–	–	–	–	–
5 days	1	2.95	17.6	117	17.9	96	28 336
	2	2.40	31.2	122	45.1	67	16 074

[a]Each experiment consisted of two rats.

[b]Experiment performed on rats receiving 2 Gy ^{60}Co TBR, dose rate 0.4 Gy/min.

between these two assays can be explained by the presence of dead and dying cells in the Ox7 upper 20% positive, Ox22 negative window or the HSC window. If these cells are either dead HSC or nonspecifically phycobiliprotein-antibody-tagged dead cells, then the number of surviving HSC as determined by the FACS assay would be overestimated. However, the total difference between the phenotypic FACS assay and the clonogenic CFU-S assay cannot be fully accounted for by the presence of dead and dying cells in the HSC window. It is conceivable that the difference that remains between the two assays after subtracting out these dead and dying cells from the total number of cells found within the HSC window represents damaged irradiated HSC that do not express themselves in the CFU-S assay [14].

The present optical bench arrangement and computer support limit us to 4-parameter cellular measurements, which are a forward light scatter measurement, two immunofluorescence measurements, and a DAPI-DNA fluorescence measurement. Therefore, enumeration of dead cells could be undertaken only in the Ox7 upper 20% positive, Ox22 negative window and not in the Ox7 upper 20% positive, Ox22 negative, W3/13 dim window. There is the possibility, although slight, that in a 3-parameter immunofluorescence analysis of the postirradiated HSC compartment, dead cells would not appear in the Ox7 upper 20% positive, Ox22 negative, W3/13 dim window. To perform this experiment, a 5-parameter flow cytometer would be needed.

The above limitations notwithstanding, the present FACS assay of postirradiation rat marrow indicates that at least 30 h is required for removal of dead and dying HSC from the marrow. At this time the initial regeneration of the HSC compartment can be observed by both the FACS (Table III) and the CFU-S assays [16]. HSC renewal, therefore, begins while there is still extensive cell death occurring in the marrow lymphocyte and granulocyte compartments. What influence this continued hematopoietic cell death has on the stromal tissues that support hematopoiesis and on regenerating HSC is an important question to be addressed if we wish to improve therapies for rapid recovery of the postirradiated marrow.

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